Essential Role of Polo-like Kinase 1 (Plk1) Oncogene in Tumor Growth and Metastasis of Tamoxifen-Resistant Breast Cancer

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Abstract

The most common therapy for estrogen receptor–positive breast cancer is antihormone therapy, such as tamoxifen. However, acquisition of resistance to tamoxifen in one third of patients presents a serious clinical problem. Polo-like kinase 1 (Plk1) is a key oncogenic regulator of completion of G2–M phase of the cell cycle. We assessed Plk1 expression in five chemoresistant breast cancer cell types and found that Plk1 and its downstream phosphatase Cdc25c were selectively overexpressed in tamoxifen-resistant MCF-7 (TAMR-MCF-7) breast cancer cells. Real-time monitoring of cell proliferation also showed that TAMR-MCF-7 cells were more sensitive to inhibition of cell proliferation by the ATP-competitive Plk1 inhibitor BI2536 than were the parent MCF-7 cells. Moreover, BI2536 suppressed expression of epithelial–mesenchymal transition marker proteins and 3D spheroid formation in TAMR-MCF-7 cells. Using TAMR-MCF-7 cell–implanted xenograft and spleen–liver metastasis models, we showed that BI2536 inhibited tumor growth and metastasis in vivo. Our results suggest that Plk1 could be a novel target for the treatment of tamoxifen-resistant breast cancer.

Introduction

Breast cancer is mostly diagnosed in postmenopausal women (1). Approximately 70% to 75% of breast cancers are luminal subtypes, which express estrogen receptor (ER). The growth and survival of ER-positive breast cancer depend on estrogen signaling, and antiendocrine therapy has been used as a standard strategy to treat luminal subtype breast cancer (2). Tamoxifen, a representative selective estrogen receptor modulator, blocks estrogen signaling by antagonizing the ER in luminal subtype breast cancer cells (3). Unfortunately, approximately one third of patients acquire tamoxifen resistance at the beginning of the treatment, and even patients who showed an initial response can later acquire resistance (4). Understanding the mechanism of resistance acquisition could uncover potential therapeutic targets for tamoxifen-resistant breast cancer. Polo-like kinase 1 (Plk1), a serine/threonine protein kinase, is a key regulator of completion of the G2–M phase of the cell cycle (5). Plk1 is highly overexpressed in various cancer cells, including gastric cancer (6), breast cancer (7), and liver (8), and is categorized as an oncogene (8). Because inhibition of Plk1 induces mitotic arrest of cancer cells, Plk1 inhibitors lead to a decrease in cell growth and an increase in apoptosis (7). In fact, volasertib (BI6727; ref. 9) has recently been approved as a therapeutic agent against acute myeloid leukemia (10). Several reports suggest that Plk1 is a possible target for the treatment of docetaxel-resistant prostate cancer (11, 12). Moreover, Plk1 inhibition enhances the efficacy of androgen signaling inhibitors in castration-resistant prostate cancer (13). Recently, kinome-wide functional screen identified the role of Plk1 in estrogen independency of ER-positive breast cancer (14). However, the potential role of Plk1 in tamoxifen-resistant breast cancer has not been studied. To mimic the clinical condition of tamoxifen resistance in breast cancer patients, we previously generated tamoxifen-resistant breast cancer cells (TAMR-MCF-7) from ER-positive MCF-7 cells by long-term treatment with 4-hydroxytamoxifen (15). Here, we report for the first time that Plk1 is upregulated in TAMR-MCF-7 cells compared with the parental MCF-7 cells, and Plk1 inhibition potently suppresses cell proliferation and tumor growth of TAMR-MCF-7 cells. We and other groups have previously reported that tamoxifen-resistant breast cancer cells acquire motile and invasive properties via epithelial–mesenchymal transition (EMT; refs. 15, 16). Our current study further reveals that Plk1 inhibition suppresses the expression of EMT marker proteins, spheroid formation, and in vivo metastasis of TAMR breast cancer cells.

Materials and Methods

Reagent and antibodies
Antibodies targeting Plk1, Cdc25c, cleaved caspase-3, cleaved PARP, Oct4, Sox2, Nanog, glycogen synthase kinase3 (GSK3)β, phospho-GSK3β (Ser9) horseradish peroxidase (HRP)–linked anti-rabbit, or anti-mouse IgG were purchased from Cell Signaling.
Technology. Antibodies for Cyclin B1, vimentin, c-Myc, β-catenin, ERK1, phospho-ERK, GSK3β, and HRP-linked anti-goat IgG were obtained from Santa Cruz Biotechnology. Antibodies for phospho-Akt (Ser473) and Akt were purchased from Signalway Antibody and other compounds, including thymidine, hematoxylin and eosin (H&E), were obtained from Sigma. BI6727 (9) and BIZ536 (17) were supplied from Medchem Express.

Cell culture
MCF-7 cells were obtained from Korea Cell Line Bank (#30022, 2005) and its short tandem repeat profile was confirmed. MCF-7 cells were cultured in DMEM, Thermo Fisher Scientific) containing 10% FBS and 1% penicillin/streptomycin solution (HyClone). TAMR-MCF-7 cells were generated according to a previously reported method (18) and cultured in DMEM containing 10% charcoal-stripped FBS, 1% penicillin/streptomycin solution, and 3 μmol/L 4-hydroxytamoxifen. Hormone-dependent T47D:A18 and hormone-independent and T47D:C42 cells were kindly donated from Dr. Miele (University of Mississippi Medical Center, Jackson, MS, 2012; ref. 19) and not authenticated in our laboratory. T47D:A18 and T47D:C42 cells were cultured in RPMI1640 medium (HyClone) containing 10% FBS and 10 ng/mL insulin. Mycoplasma test was routinely performed by Plasmo test (Invivogen) in all the cell lines used in this study.

Immunoblot analysis
Cells were lysed in lysis buffer (137 mmol/L NaCl, 20 mmol/L Tris-Cl pH 7.5, 1% Triton X-100, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 25 mmol/L β-glycerophosphate, 2 mmol/L sodium inorganic pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin). Cell lysates were transferred electrophoretically to 8% to 15% gradient SDS-PAGE with multiple gel casters (Hoefer, Inc.). Then, it was transferred to nitrocellulose paper with transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% v/v methanol, pH 8.3). Immunoblotting was done with primary and secondary antibodies, and was detected with LAS-3000 (Fuji LAS-3000, Japan) with HRP-substrate luminol reagent, and HRP-substrate peroxide solution (Millipore).

Immunofluorescence analysis
SiteClick Qdot Antibody Labeling Kits (Thermo Fisher Scientific) were used to conjugate on Cdc25c and Cyclin B1 antibodies, and immunocytochemistry was performed as described by previous report (20).

RNA isolation and qRT-PCR
Total RNA was isolated using TRIzol (Invitrogen), and cDNA was synthesized by reverse transcription using an oligo (dT) primer. The SYBR Green real-time PCR amplifications were conducted with the CFX real-time PCR detection system (Bio-Rad). The following primer sequences were used: human Plk1 sense: 5'-GCGAACCTTTTCCTGAATGA-3', antisense: 5'-AATGGCACCA-CATCTACCT-3', GAPDH sense: 5'-AGATCTCAACCGGATA-CATT-3', antisense: 5'-TCCCTCGAGATTTGTCACCGA-3'.

Real-time monitoring of cell proliferation
A total of 5 × 10^4 cells were seeded in 96-well plate, and the phase percentage of cells was scanned every 4 hours, until 68 hours by using the IncuCyte ZOOM Live Cell Analysis System (Essen Bioscience).

Apoptosis determination
A total of 5 × 10^4 cells were seeded in 96-well plate and caspase-3/7 fluorescent reagent (Essen Bioscience) was added 3 hours before BI2536 treatment. The percentage of caspase-3/7-positive cells were scanned up to 48 hours by using the IncuCyte ZOOM Live Cell Analysis System.

Cell migration assay
Cell migration was quantified by transwell migration assay. Cells were seeded in the upper chamber of the transwell plate, and the lower chamber was filled with 10% FBS-containing media. The cells were incubated at 37°C for 24 hours, fixed with formalin and methanol, and subsequently stained with hematoxylin for 10 minutes, followed by 4-minute eosin staining. With ×40 magnification, migrated cells to the lower filter side were analyzed.

Spheroid formation
A base layer of Matrigel (BD Biosciences) was overlaid in 24-well plate. A total of 10^3 or 10^4 cells/mL were suspended in 2:1 mixture of PBS and Matrigel. The Matrigel-containing cells were incubated with FBS-containing media, and on day 7 or day 10, spheroid images were taken using microscope.

Cell-cycle analysis
For double thymidine block, the cells were treated with 2 mmol/L thymidine for 15 hours, and the culture media were replaced with normal media for the release from thymidine block, and then, the cells were reincubated with 2 mmol/L thymidine for additional 15 hours. Cells were fixed with cold 70% ethanol and incubated overnight at 4°C. Ethanol was removed and the cells were resuspended in propidium iodide/Triton X-100/DNase-free RNase A staining solution for 15 minutes. The cell-cycle distribution was analyzed with FACS Calibur (BD Biosciences). A total of 2 × 10^4 cells per group were analyzed.

FACS cytometry analysis
Harvested cells were incubated with CD24-FITC, CD44-allophycocyanin, and ESA-PE antibodies (BioLegend) for 30 minutes at 4°C light-ashed condition. Immunofluorescence was assessed using FACS Calibur (BD Biosciences).

Xenograft tumor growth assay
Six-week-old BALB/c athymic nude mice (Raon Bio Inc.) were inoculated with 4 × 10^5 TAMR-MCF-7 cells. When tumors became palpable (14 days), the mice were randomly allocated to control and BI2536 treatment groups. The average of tumor volume for each groups was approximately 140 mm^3. Vehicle (20% polyethylene glycol-400, 80% normal saline) and BI2536 (10 and 20 mg/kg) were intraperitoneally injected twice a week. Tumor volumes were measured as described previously (21). All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval #: SNU-130731-1-1).

Spleen-liver metastasis mouse model
Six-week-old BALB/c athymic nude mice (Raon Bio Inc.) were inoculated with 1 × 10^6 TAMR-MCF-7 cells in spleen. Subcoastal
Role of Plk1 in Tamoxifen-Resistant Breast Cancer

A. Selective upregulation of Plk1 in TAMR-MCF-7 cells. Western blot analyses were performed to determine protein levels of Plk1 in five different sets of chemoresistant cancer cells [daunorubicin-resistant AML (AML-2/D100), paclitaxel-resistant oral cancer cells (PTX-AT-84), tamoxifen-resistant breast cancer cells (TAMR-MCF-7), paclitaxel-resistant pharynx squamous cancer cells (PTX-FaDU), and flutamide-resistant prostate cancer cells (FLTR-LNCaP)]. Data were confirmed by two repeated experiments.

B. Plk1 upregulation in hormone-resistant T47D:C42 cells.

C. Kaplan–Meier plotter analysis of correlation between PLK1 expression and relapse-free survival rate in ER-positive breast cancer patients administered with tamoxifen.

D. Plk1 mRNA determination. mRNA level of Plk1 was determined in MCF-7 and TAMR-MCF-7 cells by qRT-PCR. Data represent mean ± SD with six different samples.

P < 0.005, significantly different from MCF-7 cells.

E. Upregulation of Plk1 and its downstream targets in TAMR-MCF-7 cells. Protein levels of Plk1, Cdc25c, and Cyclin B1 were determined in MCF-7 and TAMR-MCF-7 cells by Western blotting. Data represent mean ± SD with four to seven different samples. ***, P < 0.005; **, P < 0.01; *, P < 0.05, significantly different from MCF-7 cells.

F. Cell-cycle progression of MCF-7 and TAMR-MCF-7 cells. Cell-cycle distribution was measured in MCF-7 and TAMR-MCF-7 at the indicated time points after release from double thymidine block.

Figure 1.

Plk1 upregulation in tamoxifen-resistant breast cancer. A, Selective upregulation of Plk1 in TAMR-MCF-7 cells. Western blot analyses were performed to determine protein levels of Plk1 in five different sets of chemoresistant cancer cells [daunorubicin-resistant AML (AML-2/D100), paclitaxel-resistant oral cancer cells (PTX-AT-84), tamoxifen-resistant breast cancer cells (TAMR-MCF-7), paclitaxel-resistant pharynx squamous cancer cells (PTX-FaDU), and flutamide-resistant prostate cancer cells (FLTR-LNCaP)]. Data were confirmed by two repeated experiments. B, Plk1 upregulation in hormone-resistant T47D:C42 cells. C, Kaplan–Meier plotter analysis of correlation between PLAT expression and relapse-free survival rate in ER-positive breast cancer patients administered with tamoxifen. D, Plk1 mRNA determination. mRNA level of Plk1 was determined in MCF-7 and TAMR-MCF-7 cells by qRT-PCR. Data represent mean ± SD with six different samples.

***, P < 0.005, significantly different from MCF-7 cells.

E, F. Plk1 and its downstream targets in TAMR-MCF-7 cells. Protein levels of Plk1, Cdc25c, and Cyclin B1 were determined in MCF-7 and TAMR-MCF-7 cells by Western blotting. Data represent mean ± SD with four to seven different samples. ***, P < 0.005; **, P < 0.01; *, P < 0.05, significantly different from MCF-7 cells.

F. Cell-cycle progression of MCF-7 and TAMR-MCF-7 cells. Cell-cycle distribution was measured in MCF-7 and TAMR-MCF-7 at the indicated time points after release from double thymidine block.
incision (10 mm) was made to expose the spleen. The half of spleen was tied with nylon thread to prevent leakage of injecting cells. The suspended cells were injected into the spleen, and the spleen was restored back into abdominal cavity. The incisions were closed with nylon thread. After 14 days, 10 mg/kg BI2536 was intraperitoneally injected twice a week for additional 4 weeks. The procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval #: SNU-140515-2).

Statistical analysis
Statistical comparisons between the groups were analyzed using the Student t test. The level of statistical significance was represented (*, *P < 0.05; **, *P < 0.01; and ***, *P < 0.005).

Results

Plk1 upregulation in tamoxifen-resistant breast cancer cells

Previous studies have shown that Plk1 is overexpressed in chemoresistant prostate and pancreatic cancer cell lines (daunorubicin-resistant AML, AML-2/D100, paclitaxel-resistant oral cancer cells [PTX-AT-84], tamoxifen-resistant breast cancer cells [TAMR-MCF-7], paclitaxel-resistant pharynx squamous cancer cells [PTX-FaDu], and flutamide-resistant prostate cancer cells [FLTR-LNCaP]) compared with their parent cells. Among five chemoresistant cancer cell sets, Plk1 was only overexpressed in TAMR-MCF-7 cells compared with control MCF-7 cells (Fig. 1A). We further examined Plk1 expression in another tamoxifen-resistant clone, T47D:CA2 cells, in which ER function was irreversibly eliminated by overexpression of ERα by a retroviral vector. TAMR-MCF-7 cells were incubated with 1 nmol/L BI2536 and 4-hydroxytamoxifen (1, 3, or 10 μmol/L) for 24, 48, and 72 hours. Data, mean ± SE (n = 8). *, *P < 0.05; **, *P < 0.01; and ***, *P < 0.005, significantly different from vehicle-treated control.

Figure 2.

Effect of Plk1 inhibition on cell proliferation of TAMR-MCF-7 cells. A, Effects of BI2536 on Plk1 signaling in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with BI2536 (1, 5, and 10 nmol/L) for 24 hours, and total cell lysates were subjected to immunoblottings for Plk1, Cdc25c, and Cyclin B1. B, Immunocytochemistry of Cdc25c and Cyclin B1. C, Immunofluorescence was measured 24 hours after incubation with BI2536 (1 and 5 nmol/L) in TAMR-MCF-7 cells. C, Effects of Plk1 inhibitors on cell proliferation of MCF-7, TAMR-MCF-7, T47D:A18, and T47D:CA2 cells. Left, representative Incucyte images; right, relative cell proliferation rate. Cell proliferation was determined by using Incucyte real-time monitoring system. BI2536 (1, 5, and 10 nmol/L), and BI6727 (3, 10, and 30 nmol/L) were treated for 68 hours. The representative images were taken 68 hours after the treatment. Data, mean ± SE (n = 8). *, *P < 0.05; **, *P < 0.005, significantly different from vehicle-treated control. D, Combination efficacy of 4-hydroxytamoxifen with BI2536. TAMM-MCF-7 cells were incubated with 4-hydroxytamoxifen (1, 3, or 10 μmol/L) in the presence or absence of 1 nmol/L BI2536 for 68 hours. Data, mean ± SE (n = 8). *, *P < 0.01; **, *P < 0.005, significantly different from vehicle-treated control.

To clarify the physiologic role of Plk1 upregulation in tamoxifen resistance, we used a Plk1-selective inhibitor, BI2536 (17). After exposure of TAMR-MCF-7 cells to 1, 5, and 10 nmol/L BI2536, Cdc25c expression was abolished in a concentration-dependent manner, whereas Cyclin B1 levels were increased by treatment with 5 and 10 nmol/L BI2536 (Fig. 2A). These results were confirmed by immunocytochemistry using 5 nmol/L BI2536 (Fig. 2B), which demonstrated that Plk1 was efficiently inhibited by 5 nmol/L BI2536 in TAMR-MCF-7 cells. We then assessed the effects of BI2536 on proliferation of MCF-7, TAMR-MCF-7 cells using an Incucyte real-time monitoring system for 68 hours. Significant inhibition of cell proliferation was found in TAMR-MCF-7 cells treated with 1, 5, and 10 nmol/L BI2536, and complete inhibition was achieved at 5 nmol/L (IC50 value = 2.2 nmol/L, Fig. 2C). Treatment with 5 or 10 nmol/L BI2536 also marginally inhibited the proliferation of MCF-7 cells; 10 nmol/L BI2536 inhibited cell proliferation by approximately 50% (Fig. 2C). We further showed that responsiveness of BI2536 was more sensitive in proliferation of T47D:CA2 cells than T47D:A18 cells (Fig. 2C). BI6727 (volasertib used in acute myeloid leukemia) treatment more potently inhibited cell proliferation of TAMR-MCF-7 cells than that of MCF-7 cells (Fig. 2C). We also assessed the combination efficacy of 4-hydroxytamoxifen (active metabolite of tamoxifen) with BI2536. In comparison with 4-hydroxytamoxifen (1, 3, 10 μmol/L) alone groups, cotreatment of TAMR-MCF-7 cells with 1 nmol/L BI2536 and 4-hydroxytamoxifen (1, 3, or 10 μmol/L) showed synergistic inhibition of cell proliferation (Fig. 2D). In cell-cycle analysis, the G2/M phase fraction was highly increased by incubation with 5 nmol/L BI2536 for 72 hours (Fig. 2E). Real-time monitoring of caspase-3/7

www.aacjournals.org Mol Cancer Ther; 17(4) April 2018 829

Published OnlineFirst February 7, 2018; DOI: 10.1158/1535-7163.MCT-17-0545

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Figure 3.
Effect of Plk1 inhibitor on tumor growth in TAMR-MCF-7 cell-implanted xenografts. A, Representative images for mice and tumors. TAMR-MCF-7 cells (4 × 10⁶/mouse) were flank-inoculated, and 20 days after, the mice were intraperitoneally injected with BI2536 (10 or 20 mg/kg), and tumor volume was measured by calipers. B, Inhibition of tumor growth by BI2536. Data, mean ± SE (n = 5). *, P < 0.05, significantly different from vehicle-injected control. C, Tumor weight. Tumor weight was measured on the day of sacrifice. Data, mean ± SD (n = 5). ***, P < 0.005, significantly different from vehicle-injected control. D, Number of PCNA-positive cells in tumor tissues. PCNA-positive cells were counted after IHC. A total of 5 high-power fields were analyzed in tumor tissues of individual animal. Data, mean ± SD (n = 5). ***, P < 0.005, significantly different from vehicle-injected control. E, Number of TUNEL-positive cells in tumor tissues. Data, mean ± SD (n = 5). ***, P < 0.005, significantly different from vehicle-injected control. F, Downregulation of Cdc25c expression by BI2536 in tumor tissues. Cdc25c protein levels in tumor tissues were quantified by immunoblotting. Data, mean ± SD (n = 5). *, P < 0.05, significantly different from vehicle-injected control.
activity by Incucyte further revealed that 5 nmol/L BI2536 induced apoptosis in TAMR-MCF-7 cells, suggesting that G2-M arrest and apoptosis were simultaneously induced by 5 nmol/L BI2536. However, the apoptosis index was only marginally changed in 5 nmol/L BI2536-treated MCF-7 cells (Fig. 2F). Moreover, expression of representative apoptosis markers, cleaved forms of caspase-3 and PARP, were enhanced 72 hours after treatment of TAMR-MCF-7 cells with 5 or 10 nmol/L BI2536 (Fig. 2G). These data suggest that Plk1 inhibition potently suppressed cell proliferation through G2-M arrest and eventually resulted in apoptosis in tamoxifen-resistant breast cancer cells.

**Effect of Plk1 inhibitor on tumor growth in a TAMR-MCF-7 implanted xenograft**

To determine the *in vivo* anticancer effect of BI2536, athymic nude mice bearing TAMR-MCF-7 xenografts were established. The mice were intraperitoneally injected with 10 or 20 mg/kg BI2536 twice a week (7). Tumor volume was measured on the same day as BI2536 treatment. Significant tumor growth inhibition was found in both treatment groups (10 and 20 mg/kg). BI2536 at 10 or 20 mg/kg showed significant tumor growth inhibition from 8 days after the injection, but dose dependency was not detected (Fig. 3A and B). Tumor weight was also significantly reduced by BI2536 treatments (Fig. 3C). PCNA and TUNEL IHC analyses showed a decrease in the number of PCNA-positive cells and an increase in the number of TUNEL-positive apoptotic cells in groups treated with 10 or 20 mg/kg BI2536 (Fig. 3D and E). Calc25c protein expression, a representative marker for Plk1 activity, in the tumors was significantly reduced in the groups treated with 20 mg/kg BI2536 (Fig. 3F). These results indicate that Plk1 inhibition is an effective way to inhibit tumor growth of tamoxifen-resistant breast cancer.

**Suppression of cell migration and EMT by Plk1 inhibition in TAMR-MCF-7 cells**

Tamoxifen-resistant breast cancer cells acquire motile and invasive properties. Our previous study demonstrated that chemoresistance and higher invasion phenotypes are associated with EMT induction in TAMR-MCF-7 cells (15). It has also been shown that invasion through the extracellular matrix and metastasis of breast cancer into the brain are prevented by Plk1 inhibition (24, 25). When we compared cell migration of MCF-7 and TAMR-MCF-7 cells, only TAMR-MCF-7 cells showed high migration in transwell analysis (Fig. 4A). The enhanced cell migration was potently inhibited by 1 or 5 nmol/L BI2536 (Fig. 4A). By Kaplan–Meier plot, we found an inverse correlation between PLK1 expression and distant metastasis-free survival rates in ER-positive breast cancer patients treated only with tamoxifen (n = 664; P < 0.005; Fig. 4B). To determine the *in vivo* effect of Plk1 inhibitor on metastasis, a spleen–liver metastasis model was used as described in Materials and Methods. The incidence of metastasis in liver was completely suppressed in the 10 mg/kg BI2536-treated group (Fig. 4C). H&E staining of liver tissues also revealed suppression of the liver-metastasized tumor burden in the BI2536-treated group (Fig. 4C, middle). These findings suggest that both cell migration and cancer metastasis of TAMR-MCF-7 cells are dependent on Plk1 activation.

Loss of E-cadherin and upregulation of vimentin, N-cadherin, Zeb1, and Snail are representative markers for EMT phenotype change in mammary epithelial cells (26). We therefore analyzed the protein expression of E-cadherin, N-cadherin, vimentin, Zeb1, and Snail in TAMR-MCF-7 and MCF-7 cells. As reported in our previous studies (15, 27), loss of E-cadherin and upregulation of N-cadherin, vimentin, Snail, and Zeb1 were detected in TAMR-MCF-7 cells (Fig. 4D). Incubation of TAMR-MCF-7 cells with 1 or 5 nmol/L BI2536 resulted in decreased protein expression of N-cadherin, Zeb1, and Snail in TAMR-MCF-7 cells (Fig. 4E). However, contrary to our expectation, vimentin was upregulated by 5 nmol/L BI2536. It has been reported that Akt/GSK3β and ERK pathways are activated by Plk1, and their activation induced EMT in gastric (6) and prostate cancer (28). Consistent with these reports, we found that the phosphorylations of Akt, GSK3β, and ERK in TAMR-MCF-7 cells were enhanced compared with MCF-7 cells (Fig. 4F, left). When we assessed the effects of Plk1 inhibitor on the activities of Akt/GSK3β and ERK in TAMR-MCF-7 cells, Plk1 inhibition selectively suppressed ERK phosphorylation. Although the basal level of phospho-ERK was not increased in T47D:C42 cells compared with its parental cells (Fig. 4F, left), exposure of T47D:C42 cells with 5 nmol/L BI2536 for 1 hour decreased ERK phosphorylation (Fig. 4F, middle and right). Because ERK activation is a key signaling for migration, EMT and cancer stemness of breast cancer (29, 30), Plk1-dependent phenotype changes in TAMR-MCF-7 cells may be mediated through ERK activation. These data imply that Plk1-dependent cell migration might be related to EMT in tamoxifen-resistant breast cancer cells.

**Effects of Plk1 inhibitor on cancer stemness and spheroid formation in TAMR-MCF-7 cells**

Spheroid formation of breast cancer cells under 3D conditions or on ultralow attachment plates is a hallmark of EMT and cancer stemness (31). MCF-7 and TAMR-MCF-7 cells were cultured in Matrigel precoated plates. TAMR-MCF-7 cells displayed a highly disorganized phenotype, whereas MCF-7 cells showed an acinar phenotype (27). Moreover, the number of spheroids with diameter greater than 100 μm was higher in TAMR-MCF-7 cells than in parental MCF-7 cells (Fig. 5A). Intriguingly, spheroid counts were lower in the 5 nmol/L BI2536-treated group (Fig. 5B). Moreover, mammosphere-forming ability was approximately 2-fold increased in T47D:C42 cells than in parental T47D cells, and the enhanced number of spheres was significantly diminished by 5 nmol/L BI2536 treatment (Fig. 5A and B). One of the unfortunate consequences of chemotherapy is the generation of tumor-initiating cancer stem cells (CSC; ref. 32). Recent studies demonstrated that tamoxifen induces cancer stemness, and tamoxifen-resistant breast cancers possess a CSC population (33). Because we hypothesized that the increased number of spheroids in TAMR-MCF-7 cells was due to a high CSC population, we compared the expression of several CSC transcriptional regulators, c-Myc, Sox2, Oct4, Nanog, β-catenin, and Klf4, between MCF-7 and TAMR-MCF-7 cells. The protein levels of c-Myc (3.76-fold), Sox2 (4.29-fold), and Oct4 (3.80-fold) were significantly increased in TAMR-MCF-7 cells compared with MCF-7 cells, whereas the expression of β-catenin (0.72 fold) and Klf4 (0.59 fold) was decreased (Fig. 5C). Nanog could not be detected in either MCF-7 or TAMR-MCF-7 cells. It has been reported that Plk1 inhibition suppressed the CSC population in normal...
pediatric neural stem cells and triple-negative breast cancer cells, suggesting Plk1 as a promising target for metastatic neuroblastoma and breast cancer (34–36). After exposure to 5 nmol/L BI2536, the expression levels of c-Myc, Sox2, β-catenin, and Ki67 in TAMR-MCF-7 cells were significantly diminished, but these effects were not observed for 1 nmol/L BI2536 (Fig. 5D). Previous studies have also shown that CD24−, CD44+, and ESA+ are critical membrane markers of cancer stemness in breast cancer (34). Several studies reported that Plk1 knockdown or pharmacologic inhibition suppressed CSCs via a reduction in the CD24− population in breast cancer (35, 37). To assess CSCs, we analyzed the CD44+, CD24−, and ESA+ populations in MCF-7 and TAMR-MCF-7 cells by flow cytometry and found that the CD24− cell population was increased by 10.7% in TAMR-MCF-7 cells compared with MCF-7 cells (Fig. 5E). However, CD44+ and ESA+ cell populations in TAMR-MCF-7 cells exhibited a 91.0% and 99.7% reduction, respectively, compared with MCF-7 cells (Fig. 5E). TAMR-MCF-7 cells treated with 5 nmol/L BI2536 exhibited a 20.0% reduction in CD24− cells compared with the vehicle-treated TAMR-MCF-7 cells. However, no significant shifts in CD44+ and ESA+ populations were observed after BI2536 exposure (Fig. 5E). Although membrane stemness markers are not typically expressed in TAMR-MCF-7 cells, our data demonstrate that Plk1 inhibition suppresses cancer stemness in these cells.

Discussion

Approximately 70% of breast cancers are luminal subtypes that highly express ERα (38), and ER modulators including tamoxifen and raloxifene, and aromatase inhibitors are effective therapeutic options for patients who are diagnosed with this subtype (39). Among them, tamoxifen has been the best therapeutic option to prevent or treat ER-positive breast cancer for several decades (2). Unfortunately, patients with ER-positive breast cancer who are treated with tamoxifen for more than 5 years can show acquired resistance (4). In fact, several studies have reported ER loss in 20% of patients treated with long-term tamoxifen (4). In fact, several studies have reported ER loss in 20% of patients treated with long-term tamoxifen, and these changes are consistent with Kaplan–Meier plots are at least over 33 months treated with tamoxifen, we assumed that considerable number of patients showed inherited or acquired tamoxifen resistance. We also showed that the proliferation rate of TAMR-MCF-7 cells was enhanced 1.4-fold relative to MCF-7 cells, and that the sensitivity of proliferation of TAMR-MCF-7 cells to Plk1 inhibitor BI2536 was obviously higher compared with the parental cells, suggesting that TAMR-MCF-7 cells grow in a Plk1-dependent manner. BI2536 is known as a potent dual inhibitor for Plk1 and bromodomain4 (BRD4). IC50 values of BI2536 for Plk1 and BRD4 are 0.83 and 7 nmol/L, respectively (17, 41). Because 5 nmol/L BI2536 completely suppressed cell-cycle progression and cell proliferation of TAMR-MCF-7 cells, BRD4 inhibition by BI2536 may not be related with anticancer effect of the compound in TAMR-MCF-7 cells. Plk1 is known to be a key factor in G2–M progression in cell-cycle regulation (5). BI2536 not only caused G2–M arrest, but also evoked apoptosis, as evidenced by increase in caspase-3/7 activity and cleavage of PARP and caspase-3 in breast cancer (7).

We further demonstrated that cell migration and metastasis of TAMR-MCF-7 cells were completely blocked by Plk1 inhibition, which might be related to suppression of EMT progression. Kopper and colleagues first introduced the spleen–liver metastasis system in mice as an efficient and reproducible way to determine in vivo metastasis (42). We showed that foci formation in the liver via metastasis of TAMR-MCF-7 cells from the spleen was completely suppressed by injection of BI2536.

EMT is a dynamic and reversible process that involves disassembly of cell–cell junctions, cytoskeleton reorganization, and increased cell migration/invasion (43). In agreement with our findings, mesenchymal types of NSCLC cell lines with high expression of vimentin and low expression of E-cadherin were more sensitive to Plk1 inhibition (44). Cai and colleagues also reported that Plk1 overexpression led to a reduction of the epithelial marker E-cadherin and induction of mesenchymal markers, N-cadherin, slug, and twist, in gastric carcinoma cells (6). Yuan and colleagues demonstrated that G protein–

![Figure 4](https://www.aacrjournals.org/MolCancerTher/article-pdf/17/4/833/498504/17-0545.pdf)
coupled receptor 30 (GPR30), which is related to tamoxifen resistance, causes the acquisition of an EMT phenotype in MCF-7 cells (45). GPR30 signaling upregulates β1-integrin expression and activates Akt and ERK (45), which also play important roles in tamoxifen resistance (46), and EMT progression (47). In prostate cancer cells, Plk1 is involved in EMT by activating cRaf/ERK signaling (28). Similar to these reports, we found that 5 nmol/L BI2536 inhibited ERK phosphorylation in TAMR-MCF-7 cells (Fig. 4F). We also unexpectedly found that 5 nmol/L BI2536 increased vimentin expression in TAMR-MCF-7 cells (Fig. 4E). According to the previous reports, although expression of other mesenchymal marker proteins was suppressed by resveratrol or twist knockdown, vimentin expression rather increased or was unchanged in ovarian cancer and breast cancer cells, respectively (48, 49). Hence, it would be possible that transcriptional or posttranslational control of mesenchymal marker genes may be differentially regulated by Plk1 inhibition.

CSCs are a small population of cells found within tumors that possess the capacity for self-renewal and differentiation (50). These CSCs are known as one of the main causes of tumor relapse after chemotherapy or surgical procedures (50). The mammosphere culture system was first introduced by Dontu and colleagues as a method of culturing nonadherent samples. (C3/C3) Cell surface CSC markers. CD24, CD44, and ESA were stained in MCF-7, TAMR-MCF-7, and 5 nmol/L BI2536-treated TAMR-MCF-7 cells. CD24, CD44, ESA, and ESA+ populations had completely disappeared in tamoxifen-resistant breast cancer cells. Overall, we demonstrate for the first time that tamoxifen-resistant breast cancer cells are highly sensitive to Plk1 inhibition. Because Plk1 as an oncogene is involved in multiple stages of cancer development, cell-cycle progression, migration/metastasis, and EMT through cancer stemness, we suggest Plk1 inhibition as an effective strategy to overcome tamoxifen resistance in breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
This work was supported by a grant from the National Research Foundation of Korea (2014M3A9A9073788; to K.W. Kang), Republic of Korea.

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Received June 14, 2017; revised October 17, 2017; accepted February 1, 2018; published first February 7, 2018.

Figure 5.
Effect of Plk1 inhibitor on cancer stemness in TAMR-MCF-7 cells. A, Spheroid formation in tamoxifen-resistant breast cancer cells. Left, spheroid formation in MCF-7 and TAMR-MCF-7 cells. Spheroid numbers (diameter >100 μm) were counted 10 days after seeding, and the values were expressed as relative to spheroid number of MCF-7 cells. *P < 0.05, significantly different from MCF-7 cells. Right, spheroid formation in T47D:A18 and T47D:C42 cells. Spheroid numbers (diameter >100 μm) were counted 7 days after seeding, and the values were expressed as relative to spheroid number of T47D:A18 cells. *P < 0.05, significantly different from T47D:A18 cells. B, Inhibition of spheroid formation by BI2536. TAMR-MCF-7 and T47D:C42 cells were cultured on Matrigel, and the cells were exposed to 1 or 5 nmol/L BI2536 for 7 days. Data, mean ± SD (n = 3). *P < 0.05; **P < 0.005, significantly different from vehicle-treated control. C, Upregulation of CSC markers in TAMR-MCF-7. c-Myc, Sox2, Oct4, Klf4, and β-catenin expression levels were determined by immunoblot analyses in MCF-7 and TAMR-MCF-7 cells. Data represent mean ± SD with three different samples. *P < 0.01; **P < 0.005, significantly different from MCF-7 cells. D, Inhibition of CSC marker expression by BI2536. TAMR-MCF-7 cells were incubated with vehicle or BI2536 (1 and 5 nmol/L) for 24 hours, and the protein levels of C-Myc, Sox2, Oct4, Klf4, and β-catenin were quantified. Data represent mean ± SD with three different samples. *P < 0.05; **P < 0.005, significantly different from vehicle-treated control. E, Cell surface CSC markers. CD24, CD44, and ESA were stained in MCF-7, TAMR-MCF-7, and 5 nmol/L BI2536-treated TAMR-MCF-7 cells. CD24, CD44, and ESA populations in each group were assessed by using flow cytometry. Data represent mean ± SD with three different samples. *P < 0.05; **P < 0.005, significantly different from vehicle-treated control.
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Mol Cancer Ther 2018;17:825-837. Published OnlineFirst February 7, 2018.

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